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4. Introduction

When this project began in 1999, during the first year of the grant, the ultimate goal of genetic therapy as conceived at the time was to permanently introduce new DNA into a patient's somatic cells to express a therapeutic gene product. Much of the excitement about gene therapy in recent years originates from spectacular successes in understanding the pathomechanisms of mutations in disease genes, together with rapid technological advances in cell culture and manipulation, tissue engraftment and methods of gene transfer and delivery. In the skin, much of this progress has stemmed from understanding the structural features of the epidermis and dermal-epidermal junction, largely through molecular cloning of genes which encode proteins critical for its integrity, and has led to the definition of the molecular basis of several heritable disorders. The prototype of these conditions is epidermolysis bullosa, which manifests with blistering and erosions of the skin and mucous membranes spontaneously or as a result of minor trauma. At the ultrastructural and histological level, the blisters characteristic of hemidesmosomal subtypes of junctional EB are very similar to those induced by chemical vesicants such as sulphur mustard.

In the late 1990s, the molecular basis of the hemidesmosomal forms of JEB was established through cloning of candidate genes, delineation of pathogenetic mutations in patients, and characterization the functional consequences of mutations at the cellular level. Thus, the essential prerequisites for the beginning of *in vitro* phenotypic reversion experiments are now in place, and we believe that the skin is an ideal target organ for eventual gene delivery *in vivo* for several reasons. These include the availability of keratinocyte culture and grafting techniques, the potential for targeting stem cells, and importantly, the ability to remove a graft in the event of unforeseen complications. We proposed the following specific aims:

Specific Aim 1: Assembly of contiguous cDNA constructs for introduction of the wild-type cDNA for BPAG2 into control cells, and establishment of gene transfer efficiency. *In vitro* gene transfer efficiency will be monitored by quantitative PCR and immunodetection in a cell culture model system.

Specific Aim 2: Introduction of the BPAG2 cDNA into cultured JEB keratinocytes using nonviral gene transfer techniques. Creation of a stably transfected line of GABEB patient cells will be tested for HD-AF reconstitution potential using a recombinant skin model *in vitro*.

Significance

Chemical warfare agents such as sulfur mustard (bis [2-chloroethyl] sulfide) are potent cutaneous vesicants which cause blistering in the skin by inducing separation at the dermal-epidermal junction. At the ultrastructural and histological level, the blisters characteristic of hemidesmosomal subtypes of junctional EB are quite similar to those induced by chemical vesicants such as sulphur mustard. Although the pathoetiology of vesicant-induced blistering is not well understood, several studies in the literature point to structural components in the basement membrane as potential targets for vesication. Several of these targets, including laminins and BPAG2, are the same protein components which are missing or abnormal in patients with the hemidesmosomal subtypes of JEB. Therefore, using JEB cells as an *in vitro* model system in this study, we will develop the methodology for replacement of these proteins by gene therapy into deficient cells.

The ability to create genetically enhanced skin grafts has direct implications toward the improvement of combat readiness and increased rates of wound healing in military situations in which chemical warfare agents have been deployed. The feasibility of grafting genetically-modified keratinocytes for enhanced wound healing has recently been demonstrated using an agent which increases neovascularization in the wound bed. Keratinocyte grafts which are engineered to over-express basement membrane components could serve as a temporary, readily available, and easy to apply allograft in military situations when the initial 7-14 day window for wound healing is critical. This type of cell-based method for the synthesis and delivery of local basement membrane zone components could rapidly provide the initial scaffold essential for re-epithelialization during wound healing. **Development of this technology would thereby greatly speed the initial wound healing process, and return**

military personnel injured with cutaneous vesicants to combat-ready status faster and with an improved rate of wound closure.

Overall Goals and Hypothesis

The overall goal of our studies was to establish the feasibility of delivering cellular 'bandages' to chronically wounded skin, using JEB keratinocytes as an *in vitro* model system, and we initially sought to address the issues of sustained targeted temporal and cell-type specific expression. The next step in the transfer of this technology to chronically wounded skin would be the application of *ex vivo* gene delivery by grafting of genetically engineered keratinocytes. **It is anticipated that these results may be directly relevant to the development of novel wound-care treatment modalities which synthetically recapitulate basement membrane components, by enhanced wound healing using genetically-engineered "bandages" of keratinocytes.**

5. Body

The studies outlined in this proposal initially focused upon delivery of the BPAG2 gene to GABEB patient cells as a model for producing genetically modified keratinocytes with enhanced wound healing capabilities. The preliminary studies for application of gene therapy in patients include demonstration of *in vitro* phenotypic reversion in a model system, such as the model outlined in this proposal.

During the initial 12 month period of the project, we have been establishing the basic systems that will be used to perform the proposed tasks. Specifically, we have been focusing on creating the model systems to be used for the *in vitro* gene therapy experiments. The critical elements of this system are 1) the gene construct, 2) the gene delivery methodology, and 3) the *in vitro* skin model. We have made significant progress in the first 12 month period, and completed the construct as well as developed the quantitative PCR assay for use in monitoring transgene expression.

I. Progress in Task 1: To create a recombinant plasmid vector containing the entire coding sequence of BPAG2 (1-18 months)

a. Rationale and Experimental Design

1. Assembly of a contiguous BPAG2 cDNA construct

To create the gene construct to be used for gene replacement of BPAG2, we devised a detailed gene assembly strategy and successfully proceeded with the construction of the therapeutic gene construct, mainly relying on PCR and traditional cloning techniques. During the design preparation, we took into consideration the different requirements of the potential delivery techniques as well.

The characterization and construction of a contiguous BPAG2 cDNA involved extensive PCR synthesis and ligation steps. Novel restriction endonuclease sites were introduced into the cDNA sequence (GenBank #M91669) using primer-directed mutagenesis. The restriction sites were selected with the computer program Silent (Genetics Computing Group) and did not affect the amino acid sequence encoded by these clones.

We performed long-range RT-PCR from keratinocyte mRNA, followed by standard subcloning, sequencing and ligation steps, in addition to the inclusion of linkers to facilitate ligation as needed. Long-range PCR was performed using the EXPAND Long Template PCR System (Boehringer Mannheim) with increasing extension times. PCR generated clones were sequenced in both directions using automated sequencing on ABI Prism 310 Sequencers available to us through the Skin Disease Research Center grant in the Department of Dermatology. The contiguous BPAG2 cDNA was assembled in pBluescript for convenience during the ligation steps.

Upon completion of the contig, the ~5.9 kb insert was ligated into a plasmid containing the CMV promoter, which was provided by Dr. Elizabeth

Fenjves, in the gene therapy laboratory of Dr. Lorne Taichman at SUNY Stony Brook. Our BPAG encoding construct is referred to as pCMV-BPAG2.

2. Introduction of an immunohistochemical tag for detection of BPAG2 protein production

As an alternative to using anti-BPAG2 antibodies for immunodetection of protein produced by transfected keratinocytes, as well as to distinguish the recombinant BPAG2 from endogenous, we instead introduced a short sequence to the amino-terminal end of the construct which will enable detection by the commercially available FLAG system. The immunodetectable recombinant protein product was designed to be used for morphological studies in the reconstituted HD-AF complexes in recombinant skin models in later studies. We were assisted in the use of the FLAG system by Dr. Karima Djabali, a local collaborator in the Department of Dermatology, who had extensive experience in designing constructs with FLAG tags (Djabali, et al., 2001). This construct is referred to as FLAG-BPAG2. In addition, Dr. Djabali provided expertise in helping us with the transfection techniques into cultured keratinocytes using Lipofectamine.

3. Development of a quantitative PCR assay for pCMV-BPAG2

A quantitative PCR assay was created to monitor the relative levels of expression of the mRNA off the transgene, based on the novel restriction sites introduced into the construct during the ligation design strategy (above). These novel restriction sites were used to distinguish mRNA generated by the transgene from any detectable mRNA from the endogenous genes. PCR quantitation was carried out by restriction digestion of the PCR product with the restriction endonuclease specific for the transgene, fractionation on 1% agarose, transfer of the products to nylon filters, and hybridization using an oligonucleotide probe common to both products. After exposure to autoradiography, relative ratios were normalized and calculated following densitometric scanning.

b. Outcomes, Results and Future Directions

The assembly of the plasmid was essentially completed within the first year of the project. In addition, we succeeded in FLAG-tagging the BPAG2 construct which allowed us to follow expression and discriminate between transgenic and endogenous BPAG2. During this year, the methodologies for more sophisticated quantitation by PCR began to emerge, thus as an alternative approach we will investigate the utility of the iCycler iQ real-time PCR machine (Life Technology) or similar methods which are available on campus in a Core Facility in the Hammer Building.

II. Progress in Task 2: To transiently express the BPAG2 cDNA and monitor its expression in vitro (18-30 months)

a. Rationale and Experimental Design

As a preparation for gene transfection experiments in Task 2, we tested and compared several different available delivery techniques by performing a series of transfection experiments using reporter gene systems (such as GFP) or antibiotic selection to assess transfection efficiency. During these initial experiments, we used different cell types mainly from unaffected individuals, and tested control plasmids as well as FLAG-BPAG2. We compared and tested different non-viral methods of delivery, including cationic liposomes and macromolecules, and it appeared that Lipofectamine gave the most reproducible results. Once optimized, these experimental conditions will be used with the construct developed in Task 1 for introduction into cultured cells.

b. Outcomes, Results and Future Directions

Using these systems, we were able to accomplish moderate-to-high transfection efficiency (15-25%) with different systems and mid-to-long term gene expression (2-3 weeks), similar to published reports at that time. However, data began to emerge during this period on the use of retroviral delivery system into keratinocytes, which produced greater transduction efficiency and also resulted in longer term sustained expression. We consulted with Dr. Elizabeth Fenjves, a collaborator on this grant, who in addition to advising us on the use of pCMV, also had extensive experience in the use of retroviral methods. Using her reagents from Stony Brook, we had success with retroviral transduction of control viruses into keratinocytes and were able to reproduce her results. Thus it seemed that for longer term, higher effiency gene introduction, that viral transduction was superior to transfection. Several papers appeared in the literature during the first award period that supported this view (Seitz, et al, 1999). Accordingly, at the end of the first period we contemplated moving into a retroviral delivery system for BPAG2.

III. <u>Progress in Task 3:</u> To transfect the BPAG2 cDNA into cultured GABEB patient keratinocytes and monitor their expression in recombinant skin models (18-36 months)

<u>a. Rationale and Experimental Design</u>

To accomplish the goal of *in vitro* gene therapy, even though it was early in the grant, we began working on the *in vitro* skin model described in the proposal. We initiated extended cultures of the different cells used in the assembly of the final skin model, including normal and patient keratinocytes with EB and fibroblasts. To be able to study the characteristics of the proposed model, we first proceeded with the assembly of a model consisting of normal cells, to be used as a control during the gene replacement experiments. According to the experimental outline, we performed a two-step assembly. During the first phase, we developed a multi-layer fibroblast base, which served as an acceptor surface for the engineered keratinocytes to be delivered in the second phase. After successful attachment of the seeded keratinocytes, the cultures were elevated to the air-liquid interface to allow the multi-layer growth and differentiation of the keratinocytes and expression of the transgene.

b. Outcomes, Results and Future Directions

We will continue to make improvements in the culture model systems in the second and third award periods as outlined in the proposal for months 18-36. Several reports appeared in the 1999-2000 period which would help to shape our thinking about the nature of our delivery systems and how they might be improved, in particular the work of Seitz et al. in which gene delivery of BPAG2 (also known as BP180) was performed (Seitz et al., 1999). Please refer to Section 8 below (Conclusions) for futher discussion of our future directions at the end of the first year.

6. Key research accomplishments

In the first award period, the assembly of the plasmid was essentially completed. In addition, we succeeded in FLAG-tagging the BPAG2 construct which allowed us to follow expression and discriminate between transgenic BPAG2 and endogenous. Thus we have completed all or most of the experiments related to Task 1 (1-18 months) and have begun working on conditions for Tasks 2 and 3.

7. Reportable Outcomes

Directly related to this Award:

1. Hengge UR, Taichman LB, Kaur P, Rogers G, Jensen TG, Goldsmith LA, Rees JL, Christiano AM. (1999) How realistic is cutaneous gene therapy? Exp Dermatol. 8:419-31.

Other areas of EB Research in the Lab:

2. Cserhalmi-Friedman, P.B., Grossman, J., Karpati, S., Ahmad, W., Horvath, A. and Christiano, A.M. (1999) Identification of a *de novo* Glycine Substitution in the Type VII Collagen Gene in a Proband with Mild Dystrophic Epidermolysis Bullosa. Exp. Dermatol. 8:143-145.

3. Christiano, A.M., Crollick, J., Pincus, S. and Uitto, J. (1999) Dominant Dystrophic Epidermolysis Bullosa with Squamous Cell Carcinoma: A Molecular Study. Exp. Dermatol. 8:146-152.

4. Cserhalmi-Friedman, P.B., Tang, Y., Grifo, J.A. and Christiano, A.M. (2000) Preimplantation Genetic Diagnosis in Two Families at Risk for Recurrence of Herlitz Junctional Epidermolysis Bullosa. Exp. Dermatol. 9:290-297.

8. Conclusions and Future Directions

The initial period of this research project was successful in establishing the basic systems and materials necessary to accomplish the ultimate goal of this proposal, that of successful *in vitro* gene replacement. In particular, we completed the vector construction and began preparations for the skin models. We had particular success in the early attempts at the recombinant skin models and were easily able to recapitulate the previous work of others in establishing these important reagents.

During the first award period 1999-2000, a paper appeared in the literature by Seitz et al. (1999) which reported delivery of BPAG2 (BP180) using retroviral transduction of patient keratinocytes and containing many of the same approaches outlined in our proposal. These investigators demonstrated restoration of BPAG2 from patient keratinocytes, and subsequent normalization of the adhesion defect or keratinocytes in culture. Further, regenerated skin with transduced keratinocytes was then grafted onto nude mice and it was shown that reconstitution of a normal dermal-epidermal junction occurred in the engineered cells but not in the untransduced controls. It was very gratifying to see that our hypothesis was indeed correct, and that in fact as we and others had predicted, introduction of exogenous BPAG2 could indeed reverse the phenotype of patient skin with mutations and improve adhesion and integrity, at least in model systems.

The appearance of this work and several others around this time clearly demonstrated the feasibility of gene introduction techniques for the purposes of gene therapy for different types of EB (Bauer, et al., 1999; Khavari, 2000). **Importantly, however, these papers underscored several of the emerging difficulties of all gene therapy approaches in many tissues other than skin, including: 1) low efficiency of gene introduction; 2) lack of sustainable long-term expression; 3) inability to target the stem cell compartment and 4) the potential for immune response against the protein, which is foreign to the patient due to genetic mutations.**

Clearly all of these obstacles also bore relevance to the subject of military relevance of our application in terms of the feasibility of creating generic cellular 'bandages'. If we and others were not able to achieve long term viability of easily produced keratinocyte-fibroblast recombinant skin systems, then these technologies would represent very limited applications for only a handful of patients. Also, the utilization of retrovirus and adenovirus vectors in human gene therapy at this time also became controversial due to the unexpected life-threatening complications that arose during in vivo gene therapy trials in humans (Russi, et al, 1997; Wivel and Wilson, 1998; Harvey, et al., 1999). Thus we ended the first award period with the feeling of satisfation that our hypothesis had been validated (Seitz et al, 1999), but at the same time, with a new challenge - to devise a more universally applicable, easily accessable, non-virally mediated skin equivalent that had all of the following properties: 1) long term gene expression; 2) the presence of stem cells; and 3) lack of immune response, or conversely, a source of universal donor tissue from which engineered 'bandages' could be developed, both for the different forms of EB, but importantly, for the purpose of fulfilling the **original goals** of this award - **to establish the feasibility of delivering cellular 'bandages' to chronically wounded skin.**

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10. Appendices

Publication #1 is included.

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Controversies in Experimental Dermatology

Section Editor: Ralf Paus, Hamburg

How realistic is cutaneous gene therapy?

Hengge UR, Taichman LB, Kaur P, Rogers G, Jensen TG, Goldsmith LA, Rees JL, Christiano AM. How realistic is cutaneous gene therapy? Exp Dermatol 1999: 8: 419–431. © Munksgaard, 1999

U. R. Hengge, L. B. Taichman, P. Kaur, G. Rogers, T. G. Jensen, L. A. Goldsmith, J. L. Rees and A. M. Christiano

Recent progress with innovative, experimental gene therapy approaches in animals, and recent improvements in our understanding and manipulation of stem cells, gene expression and gene delivery systems, have raised plenty of hopes in essentially all branches of clinical medicine that hitherto untreatable or poorly manageable diseases will soon become amenable to treatment. Few other organ systems have received such enthusiastic reviews in recent years as to the chances and prospects of gene therapy as the skin, with its excellent accessibility and its pools of – seemingly – readily manipulated epithelial stem cells (cf. Cotsarelis et al., Exp Dermatol 1999: 8: 80–88).

However, as in other sectors of clinical medicine, the actual implementation of general gene therapy strategies in clinical practice has been faced with a range of serious difficulties (cf. Smith, Lancet 1999: 354 (suppl 1): 1–4; Lattime & Gerson (eds.), Gene Therapy of Cancer, Academic Press, San Diego, 1999). Thus, it is critically important to carefully distinguish unfounded hype from justified hope in this embryonal area of dermatologic therapy, to discuss in detail what can be realistically expected from cutaneous gene therapy approaches in the next few years, and importantly, what kind of promises should not be made to our patients at this time.

Viewpoint 1

Gene therapy is a new field of biotechnology that deals with treating diseases with DNA. While early gene therapy was confined to hematopoietic cells, the skin has rapidly become a major organ for genetic manipulations due to its accessibility and immunological properties. Both skin fibroblasts and epidermal keratinocytes have been employed as targets. In order to appreciate its value, it is important to recall the different scenarios in which skin gene therapy can be helpful (Fig. 1).

Skin as a bioreactor
Skin as a metabolic sink
Skin as a vaccination organ
Skin treatment

Figure 1. Various scenarios for skin gene therapy.

First, the skin can serve to synthesize various proteins that have therapeutic value, either systemically or locally. In this regard, the first systemic corrective genetic approach was performed in 1987 with transduced fibroblasts or transduced autologous lymphocytes from adenosine-deaminase-deficient humans for severe combined immunodeficiency (SCID) syndrome using retroviral vectors (1, 21). At the same time, human epidermal grafts were obtained from human growth hormone-transduced keratinocytes (20), or from human apolipoprotein E (apo E)-transfected keratinocytes grafted onto athymic rats (9). In these studies, the formation of a differentiated epidermis with genetically modified keratinocytes and the continuous production of therapeutic proteins that gained access to the bloodstream have been demonstrated. Soon thereafter, hemophilia B was phenotypically corrected in dogs using retroviral vectors (10, 14).

Rosenberg and colleagues (25) were the first to perform a clinical gene-marking trial in stage IV melanoma patients. Subsequently, gene therapy of cancer in several animal models showed promising results, using IL-6, IL-2 and IFN-γ (29).

Furthermore, skin gene therapy can be used for the local treatment of genetically determined skin diseases (genodermatoses). In this regard, the increasing knowledge of genetic mutations has helped to understand a substantial number of skin diseases involving the basement membrane, and thus has allowed the development of gene therapy strategies. Recent examples of corrective skin gene therapy approaches include the assembly of hemidesmosomes in reconstructed epithelia from junctional epidermolysis bullosa patients (33), and the generation of a functional epidermal barrier in lamellar ichthyosis (4).

Second, the skin can be used to detoxify metabolic products, if the necessary enzymes are synthesized ("metabolic sink") (23). For example, enzymes such as ornithine-delta-aminotransferase can be produced in the skin, and can serve to clear hyperornithinemia which causes gyrate atrophy (28).

Third, the skin has potent immunization potential. This property was realized when *in vivo* gene transfer became possible through the introduction of the "gene gun", which allowed the transfer of genes coated onto fine gold particles into a variety of mammalian tissues and cells both *in vitro* and *in vivo* (13). Subsequently, the direct injection of naked plasmid DNA was established, thereby eliminating the need of expensive technical devices (12, 36). Potent cytotoxic T cell and humoral immune responses can be generated, exploiting antigen processing and presentation in the skin and muscle. Direct transfection of antigen-presenting Langerhans cells has been shown to vigorously stimulate immune responses (5). In addition, proteins which are produced in keratinocytes can be phagocytozed and presented in the MHC class I pathway and lead to CTL priming (7).

It has also been found that the balance between a T_{h1}- and a T_{h2}-type immune response can be influenced by choosing the organ that is employed for vaccination (e.g. skin vs muscle) and the method of gene transfer (direct injection vs gene gun) (32). In particular, injection of plasmid DNA led to a T_{h1}-immune response (IgG_{2a}), whereas particle bombardment created a predominant T_{h2}-type response (IgG_1) (8, 32). These intrinsic differences need to be kept in mind when gene therapy strategies are designed. For example, Balb/c mice

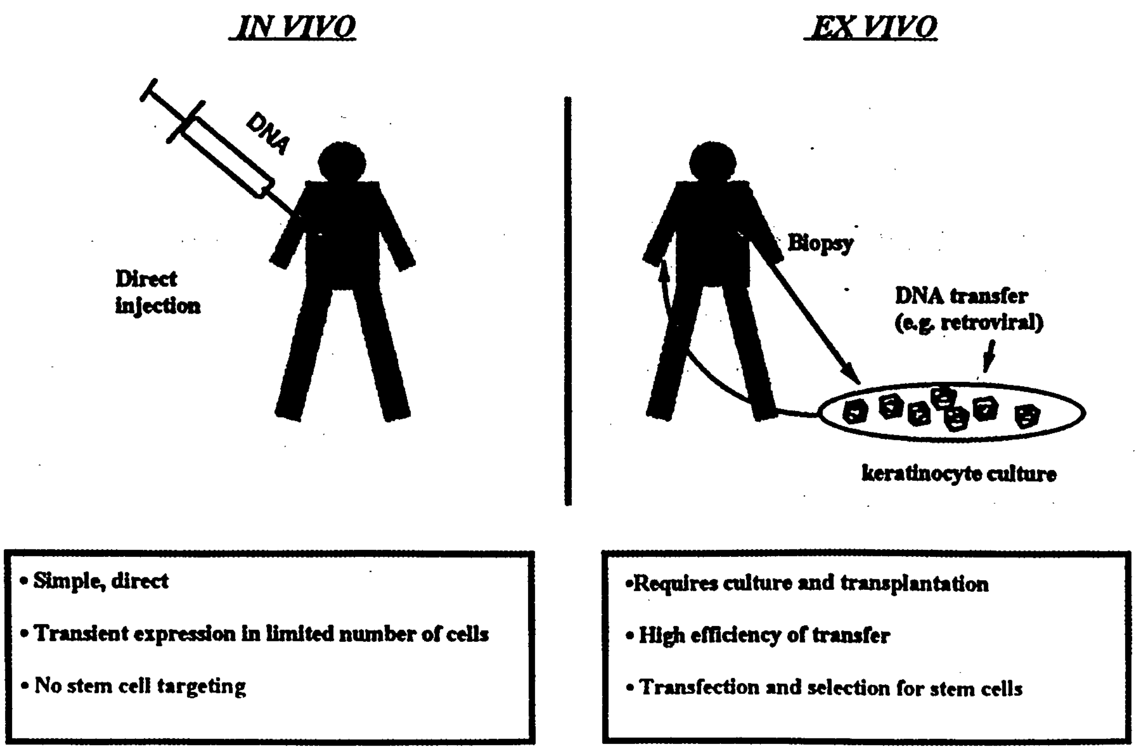

Figure 2. In vivo and ex vivo gene transfer.

Table 1. Achievements and progress in gene therapy

Improved transduction rate by pseudotyping
Vector targeting to certain tissues (target cell recognition)
Vector targeting to epidermal layers (K14, K10)
Bicistronic expression
Reduced immunogenicity of virus vectors
Phenotypic correction of disease

are susceptible to leishmaniosis, a parasitic infection associated with a T_{h2} predominance. By direct intradermal injection of a surface glycoprotein cDNA (gp-63 cDNA), a T_{h1}-type immune response is induced, which can prevent the disease (34). In addition, the administration of cytokines and co-stimulatory molecules can further influence the type of the immune response (31). Recently, the epidermal route of genetic immunization has been compared with muscular administration (11). Higher rates of seroconversion, higher antibody titers and increased cytotoxic T-lymphocytes have been detected following the epidermal mode of immunization.

Two basic approaches can be pursued to transfer genes in gene therapy (Fig. 2) (13). The *ex vivo* approach introduces genes while cells or tissues are being propagated in culture. This approach is complicated and requires laborious culturing, but allows the transfer of genes into large numbers of early progenitor/putative stem cells, which can be selected for the presence of the introduced gene. This approach has generally been used with retroviral vectors. In contrast, the *in vivo* gene transfer (i.e. gene gun, direct injection) is straightforward, but is not practical for larger skin areas or persistent expression, since stem cells are generally not transfected. In essence, the *ex vivo* setting is suitable for corrective, long-lasting gene transfer, whereas the *in vivo* techniques are being used for immunization, where transient expression is desired.

Various refinements have been achieved in vector technology and application procedures (Table 1). The advantages of plasmid DNA for immunization purposes were soon recognized, such as repetitive administration without adverse immune responses. Large gene products can be synthesized from plasmids, which is possible neither with viral transfer (due to limited capacity) nor with recombinant protein technology (due to difficulties in maintaining native conformation and glycosylation). Whereas recombinant protein is expensive and unpredictable, DNA production is cost-effective and nucleic acids can be obtained in large amounts. Consequently, several attempts have been made to facilitate gene transfer by simply applying the genetic remedy topically to the skin. Towards this end, liposomes, adenovirus, retrovirus, puncturing, jet injection, scratching or tape-stripping have proven the innovative capacity of skin researchers, but have not gone beyond proof of principle.

The novel utilization of the integument for gene therapy is due to the recently discovered characteristic of keratinocytes and Langerhans cells to take up DNA. Plasmid DNA is a large, highly negatively charged molecule and usually is found in the nucleus and mitochondria. At this point, it is still obscure how and why keratinocytes (and various other cells) take up DNA and translate it into the corresponding protein. Current experiments aim to identify DNA-binding molecules on the cell membrane, which might help to understand the mechanism of uptake. Such an understanding will ultimately offer the potential to optimally exploit this ability. This understanding will also enable researchers to more comprehensively evaluate safety aspects and biocompatibility issues associated with this technology.

Very recently, the therapy of tumors in estab-

Table 2. Examples of skin gene therapy

Clinical application	
Metastatic melanoma	Rosenberg et al. 1990 (25); Nabel et al. 1993 (22), Klatzmann et al. 1998 (15); Sun et al. 1998 (30); Schreiber et al. 1999 (26)
Hemophilia B	Lu et al. 1993 (17)
Head and neck squamous cell carcinoma	Wollenberg et al. 1999 (37)
Corrective skin gene therapy of epidermis	
Junctional epidermolysis bullosa	Vailly et al. 1998 (33), Seitz et al. (27)
Lamellar ichthyosis	Choate et al. 1996 (4)
Correction of systemic deficits using skin cells	
Adenosine deaminase	Palmer et al. 1987 (23)
Human growth hormone	Morgan et al. 1987 (20)
Apolipoprotein E	Fenjves et al. 1989 (9)
Mucopolysaccharidosis type VII (β-glucuronidase)	Moullier et al. 1993 (21)
Fabry's disease (α-galactosidase)	Medin et al. 1996 (19)

Table 3. Challenges for the next decade(s)

Tissue and cell-cycle-specific targeting
Longevity of expression/correction (manipulation of stem cells)
In vivo regulation of gene expression (tetracycline repressor/ecdysone)
Control of immune responses
Excisional repair of transdominant negative mutations
Correction of entire diseased organ (*in vivo* selection)
Topical application ("gene cream")
Safety and biocompatibility
Public acceptance

lished tumor models has directly compared protein and DNA-induced therapy (24). Treatment with IL-12 cDNA showed similar antitumor effects, but exhibited fewer side effects than treatment with IL-12 protein. This important study is the first to show the clinical equivalence of plasmid DNA with protein in cancer therapy and revealed significantly less toxicity for the DNA approach.

Since the first therapeutic experiments in the late 1980s, more than 250 additional clinical gene therapy trials were approved and more than 2000 patients were treated worldwide through the end of 1996 (Table 2 and ref. 18). The majority of the trials aimed to treat cancer employ suicide genes or immunization strategies. Since melanoma is one of the most immunogenic tumors, it represented a favorable target for gene-modified cancer vaccines (Table 2). Based on animal tumor models, a number of clinical protocols have been developed to treat cancer patients with irradiated allogeneic or autologous melanoma cells modified with various cytokine genes such as IL-2, IL-4, IL-7, IL-12, GM-CSF, IFN-γ or co-stimulatory molecules like B7.1 and B7.2 (13).

Whereas the therapy with irradiated genetically modified tumor cells is widely accepted from a safety standpoint, the utilization of plasmid DNA is currently being discussed controversially due to potential secondary effects resulting from long-term, low level expression eventually leading to tolerance or autoimmunity. Towards this end, several clinical trials have confirmed the safety of naked DNA in humans. Besides therapeutic and prophylactic vaccinations against HIV and malaria (3, 35), plasmid DNA was evaluated for immunization against CEA-expressing colon carcinoma (6), and for the treatment of arterial occlusive disease of the myocardium and leg (16).

Despite the successes, several challenges exist which have not yet been resolved (Table 3). While transferred vectors persisted, the expression of the transgene was gradually inactivated. Moreover, control of highly regulated genes seems critical, since (e.g.) transfer of the CD40 ligand into bone marrow led to constitutive expression, causing lymphoma in animal models (2).

Under critical evaluation cutaneous gene therapy has passed its infancy, and has demonstrated proof of principle. Several technical limitations in the transfer from the culture flask to the experimental animal model and, finally, onto the clinical stage have yet to be mastered. Cutaneous gene therapy certainly merits appreciation, when the relatively small number of laboratories, the limited financial support and its young age (about 12 years) are taken into consideration. The most significant progress has been achieved with DNA immunization, which allows the endogenous production of proteins from tumors and infectious agents and elicits potent antitumor or antiinfective immunity. Skin gene therapy has not yet cured humans, but has achieved beneficial clinical responses and has prolonged lives.

Especially for genetic vaccination, skin gene therapy will become a clinical reality in due course.

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Viewpoint 2

It's been five years since many of us promised our patients and families with severe inherited skin disorders that by now, clinical trials for genetic therapies in a number of diseases would be well underway.

Yet on the eve of the millennium, despite the fact that the molecular bases for a wide range of genodermatoses have been worked out in breathtaking detail, we are still promising our patients a genetic remedy "within five" years.

Haven't we been here before?

Have we unknowingly offered false hope?

We were deeply sincere five years ago when we made these statements.

So, why has the reality not lived up to the promise?

Many of the patient advocacy organizations have begun to ask these very same questions to the scientists and clinicians who comprise their Scientific Advisory Boards.

Where's the gene therapy?

In response to this challenge, one advocacy group, DEBRA, the Dystrophic Epidermolysis Bullosa Research Organization of America, Inc., together with its international counterpart, has organized a Visioning Meeting in the Fall of 1999. The concept behind this meeting is to ask the really difficult questions of its scientists, and using invited expert gene therapists from other fields as moderators, to formulate a plan for where we would like to be in 2005. It promises to be quite the event, and as a DEBRA board member and an Editor of this Journal, I hope to communicate the essence of the proceedings, perhaps as a follow-up to this Controversies.

And hard questions they shall be.

Among the most challenging are the following...

Have we chosen rational disease targets and manageable genes?

One of the cruel paradoxes of gene therapy for inherited skin disorders is that the diseases we find the most compelling are the ones that offer the greatest clinical and technical challenges. For example, recessive dystrophic epidermolysis bullosa (EB) would certainly come to mind as a disorder in need of gene therapy. The molecular basis is unequivocally established, and it ranks among the most devastating of all genodermatoses. Yet, the gene, type VII collagen, is among the largest in the literature, making delivery in size-restricted vectors a challenge. Further, in the milieu of scar tissue in dystrophic EB, where would we even locate a stem cell for grafting?

Or consider Herlitz junctional EB (HJEB). Again, the neonatal lethal course of the disease compels us to search for a therapy. However, is a disease with such extensive internal involvement really a rational target for gene delivery? Could we possibly hope to rescue a phenotype as pleiotropic as HJEB? Further, could we expect to reconstitute functional laminin or collagen trimers in a temporally appropriate and cell-type specific fashion in order to effect and regulate a gene delivery strategy? In as short a window of therapeutic opportunity as a few weeks?

Might the milder forms of these diseases offer more rational targets? For example, would EB simplex or dominant dystrophic EB be better immediate targets, by selective inhibition of the mutant alleles? Or, would GABEB offer a better alternative for working out a paradigm for gene delivery, since type XVII at least offers a smaller and more manageable gene? Clearly, choosing both a disorder where patients are less severely affected together with a relatively small gene and clever delivery strategy might optimize the chances for success.

Have we identified the best strategies for prevention and treatment relative to the underlying types and combinations of mutations?

The major goals of managing genetic disease are no different than those for an acquired disease: prevention where possible, and treatment when not.

For genodermatoses, the prevention side of the equation has recently reached the forefront of disease prevention in the form of the availability of preimplantation genetic diagnosis (PGD) for HJEB. In this procedure, following *in vitro*-fertilization, single-cell DNA diagnosis is performed from 8-cell embryos and only the healthy or carrier embryos are transferred back to the mother to establish a pregnancy (see Review Article by McGrath & Handyside in Exp Dermatol 1998: 7: 65–72). PGD offers diagnosis prior to pregnancy, thereby obviating the need for termination altogether. It is truly the ultimate step in disease prevention as we know it.

On the treatment side, we need to keep in mind that alternative strategies to total gene replacement may offer more efficacious therapeutic approaches. For example, the use of homologous recombination for gene correction is being explored. Antisense and ribozyme technologies for gene inhibition may be applicable for the dominant disorders. And finally, methods of delivery are constantly being refined. *Ex vivo* versus *in vivo*. Grafting versus gene gun. The list goes on and changes daily. However, because of the accessibility of the skin and the long-established methods for culture of cells and grafting, we should find ourselves well-positioned when vectors and enabling technologies become available.

And importantly, where is the field of EB relative to the rest of genetic diseases in terms of progress toward gene therapy?

This is the good news! We are not alone in skin disorders in promising our patients gene therapies. In fact, geneticists in almost every field of medicine have made similar promises. No one could have anticipated the obstacles that have plagued the cystic fibrosis clinical trials for example – for more than five years. No one could have predicted that the NIH would take a step back and invest heavily in vectorology for gene delivery – one of the most precarious of the technical stumbling blocks. No one could have anticipated the difficulties faced by many investigators in maintaining long term *in vivo* gene delivery. Many disciplines, dermatology included, are still working furiously to find the elusive stem cell.

As a field, we are exactly where we should be – with the molecular bases of our target disorders well in hand, hard at work in our laboratories, and poised for action as the vector and delivery technology evolves.

What we have learned then, in five years, is that there is a great deal of insight and understanding needed after we've worked out the genes in meticulous detail.

What we have learned is that Nature does not yield her secrets easily, and that getting genes into the skin is not going to be as easy as we had anticipated.

Our hopes were as high as those of the patients we serve.

We share their disappointment, although these five years have not been without heroic efforts on our part. We believe, nonetheless, that the future looks brighter than ever.

Perhaps by choosing the right course for the right horse, this time we can live up to the reality of "within five years".

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Commentary 1

The question posed by the title of these reviews, "How realistic is cutaneous gene therapy?" is somewhat misleading because it presupposes that cutaneous gene therapy is one entity with one outcome, when in fact cutaneous gene therapy is a collection of therapeutic approaches that have gene transfer to skin and its cells as a common element. As Ulrich Hengge correctly points out, cutaneous gene transfer encompasses a variety of approaches including viral and nonviral methods for *ex vivo* and *in vivo* gene transfer.

It has been my experience that a more fruitful examination of cutaneous gene therapy can be obtained if one divides applications in 2 groups, those requiring transient expression of the therapeutic gene and those requiring long term expression.

Short term transgene expression is useful for such applications as DNA vaccination whether for cancer immunization or for prevention of an infectious disease. For these applications, as Hengge explains, cutaneous gene therapy is well advanced. Nonviral methods for gene transfer are relatively simple and achieve consistent short term expression. A number of clinical gene trials using nonviral gene transfer to skin as a route of vaccination are underway and there is solid evidence from preclinical studies to be optimistic of the outcome.

Long term expression is another story. Long term transgene expression would be needed to treat inherited skin diseases and systemic diseases amenable to correction by a cutaneous source of secreted gene product. For long term expression, the only feasible method for gene transfer is the use of a retroviral vector because of its capacity for integration into the host chromosome. Long term expression has been difficult to achieve, but several inroads into this problem have been made.

First, modification of the promoter within a retroviral vector has helped to enhance and perhaps prolong expression (1, 2). However, in none of these studies have data been presented directly showing enhanced or prolonged transcription from the putative transcriptional initiation site. Without direct examination of promoter activity in the *in vivo* tissue, it is difficult to rule out other factors such as differences in grafting techniques or enhanced transduction of stem cells.

A second inroad has been the successful transduction of keratinocyte stem cells in culture. This was pointed out in two recent studies (3, 4). However, the importance of efficient stem cell transduction (5) has not been fully appreciated. Efforts to improve *in vivo* performance through modification of the enhancer/promoter complex may not be effective if only a small number of stem cells are successfully transduced. Even if the promoter regulating the transgene is highly active, if only a small percentage of stem cells in the tissue are transduced, transgene expression levels in the grafted animal will be low.

There is an additional problem underlying many studies of long term expression, and this relates to difficulties in securing stable grafts in immunocompromised animals. Long term expression studies require that *ex vivo* modified cells be accommodated for extended periods of time in an *in vivo* environment. We are now able to achieve long term grafts of human keratinocytes and fibroblasts routinely in athymic mice for observation periods as long as 40 weeks, but this capability did not come easily. It required considerable commitment of time and resources as well as the collaborative efforts of scientists in other labs to optimize raft cultures and grafting procedures. It has been our experience that when grafts are unstable, that is, they undergo contraction, or are invaded by mouse epithelial cells, or become inflamed, transgene expression is also unstable and is likely to be lost. We do not know the mechanism underlying this instability but a lack of sustained expression in an unstable graft poses a particularly difficult set of results to interpret. Investigators attempting to study long term *in vivo* expression will need to develop methods for securing stable grafts in a routine way.

In his review, Dr Hengge notes that there are no *in vivo* gene transfer methods that achieve long term expression. This was correct at the time of writing, but most recently a paper from our laboratory has appeared (6) describing direct, *in vivo* transduction of interfollicular and follicular keratinocytes with high titer retroviruses with long term, sustained transgene expression. The ability to transduce epidermis and hair follicles directly may enable corrective gene transfer without the necessity for surgical placement of *ex vivo* modified autologous cells and the attendant complications that follow such a procedure. We believe this to be an important advance for cutaneous gene therapy.

Angela Christiano poses a more difficult question than feasibility of cutaneous gene therapy. Dr Christiano asks what genodermatoses could we realistically hope to treat, including the severe, widespread, crippling disorders such as recessive dystrophic EB or Herlitz junctional EB, or the milder forms such as EB simplex. This is a more difficult question because the milder forms, though more amenable to therapy, are less likely to require intervention, while the more severe forms are overwhelming in their need for intervention, but are unlikely to be ameliorated in a significant way by a gene-based therapy.

This is truly a dilemma for which there is no simple answer. I would think we aim for the more severe forms in our research goals, but in practice we treat the less severe forms, at least in initial therapy trials. We need to explain this to patient advocacy groups. Although these groups are anxious for relief, they have shown a generosity of spirit, a strong desire to understand their affliction, and are likely to be more accepting of our limitations than we are.

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Commentary 2

Much has been said about the suitability of skin as an ideal vehicle for gene therapeutic approaches utilizing genetically manipulated keratinocytes to introduce foreign gene products for local or systemic delivery to the body. The feasibility of this approach has begun to be addressed experimentally and resulted in the identification of important problems as discussed in Viewpoints 1 & 2. One of the recurring issues that I want to discuss is the requirement for stem cell transduction to achieve lasting therapy, a view that I subscribe to while keeping an open mind about the feasibility of doing so efficiently and without losing these cells to differentiation during *ex vivo* manipulation.

The questions pertaining to the manipulation of keratinocyte stem cells for cutaneous gene therapy are "Can we isolate these cells?"; "Can we transduce them efficiently?"; "Can we maintain stem cell properties during these manipulations?"; "Will we achieve lasting therapy when we have overcome all these hurdles?"

Speaking from experience, at present it is certainly possible to obtain small numbers of stem cells using cell sorting techniques. It would be highly desirable to develop methods such as panning or magnetic selection that would permit the isolation of larger numbers of these cells, since currently it is clear that stem cells have a limited lifespan in culture making *ex vivo* expansion difficult. However, studies aimed at defining factors involved in self-renewal of stem cells may provide a means of achieving controlled expansion and transduction of these cells – after all, stem cells cycle and return to quiescence *in vivo.*

Alternatively, let us consider whether we need to go to the trouble of isolating stem cells to transduce them? Data from many laboratories show that cells can be cultured transduced en masse and transplanted onto mice, giving rise to fully formed epithelia (1–5). Kolodka et al. (5) provide compelling evidence that transplanted human keratinocytes transduced with retroviral vectors show continued expression for almost a year *in vivo.* The question then is whether the frequency of stem cells transduced in mass cultures (i.e. without purification of stem cells) is sufficient for therapeutic purposes. To this end, it is worth noting a study by Wang et al. (6) who reported that grafting a piece of tail skin from a transgenic mouse producing human growth hormone (hGH) under the control of the K14 promoter, onto a normal recipient resulted in secretion of this hormone, but not at physiological levels, despite expressing hGH in all basal keratinocytes. This is where the development of better vectors will have a major role to play in gene therapeutic approaches.

To live up to the challenge of "Controversies in Dermatology", I would like to put a twist on our assumptions that stem cells must be transduced for long-lived therapy. This seems a reasonable notion given that, *in vivo*, other proliferative cells are short-lived, and if one assumes a stem cell is inherently a unique and irreplaceable cell. However, it appears that even K10 positive keratinocytes which have initiated differentiation are capable of proliferating for several months in culture (7), and indeed revert phenotypically to transit amplifying cells expressing high levels of $\alpha_6\beta_4$ integrin (Li & Kaur, unpublished data).

I would like to speculate that, although stem cells are indeed unique and special *in vivo,* the epidermis may be a more plastic tissue than suspected, should the demand arise. What I am suggesting is that a stem cell is only a stem cell in the right milieu composed of neighboring transit amplifying cells and underlying connective tissue with a role for both endogenous and external regulating factors, such as growth factors and extracellular matrix components. It may then be possible for us to culture keratinocytes from a patient, genetically modify these and graft them back – with re-establishment of stem cells, transit amplifying cells and their differentiated progeny *in situ*! This plasticity model also provides an explanation for the observation that manipulation of cells *in vitro* and regrafting them results in reformation of a normal epithelium. It is also completely in line with our own recent data which indicates that stem cells are not the only cells capable of forming an epidermis (Li & Kaur, unpublished data).

It may just be a fantasy, but wouldn't it be a great outcome for gene therapy if there was no requirement for stem cell isolation or manipulation? Wouldn't it be incredible if the epidermis was a unique tissue in terms of its plasticity and great potential for growth? This would truly make the epidermis an ideal candidate for gene therapy. Of course, experimentation to elucidate the facts is required to determine the appropriate way to proceed.

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Commentary 3

The increasingly rapid advances in our knowledge of skin are exciting to observe and a major spin-off over the last 10–15 years is the development, in principle, of methods for cutaneous gene therapy. Their application is in two main directions. One is to use the skin as a therapeutically useful bioreactor to secrete normal products and replace defective proteins in a range of genetic disorders, and the other is more focused, the treatment of genetic conditions of the skin.

Ulrich Hengge gives a comprehensive survey of the cutaneous gene therapy literature in the broad perspective and reviews the many possible ways, *ex vivo* or *in vivo*, systemic and local, of utilizing the skin route for a range of therapies. Animal models of cutaneous gene therapy are abundant in the literature but significant human trials have not yet occurred.

The skin is the largest organ in the body with both keratinocytes and fibroblasts as major components. It would appear to have enormous potential especially via the remarkably versatile keratinocyte that normally manufactures and secretes a wide range of proteins, from cytokines to basement membrane components. The skin is more readily monitored than other organs and its use, as a kind of pseudo-liver ("metabolic sink") would seem to be particularly powerful.

Problems lie in the targeting of genes to epidermal stem cells and vectors for the stable transfection of keratinocytes are essential for optimal therapy. Retroviral vectors are efficient, but could carry unpredictable consequences for the phenotype. Plasmids can be used, but transfection is inefficient. Hence, much research needs to be done to deal with these problems including the control of expression of the transgene. Nonetheless, we should remain optimistic that there will be some degree of success in the next few years and a longer-term possible solution is discussed later.

Angela Christiano confronts the BIG challenges of the genodermatoses, especially the diverse EB group, and as she points out, promises of therapies made to patients have not been fulfilled. Equally, therapies for genetic diseases in general such as cystic fibrosis, have been disappointing. For the genodermatoses, the strikingly-rapid delineation of the molecular causes of the bullous diseases plus the equally-rapid development of DNA manipulation, stem cell identification and DNA delivery systems placed investigative dermatologists into an optimistic (but reasonable) attitude that treatment modes would be around the corner. The negative press also may have subdued the enthusiasm of funding providers and that needs to be addressed.

One can agree with Angela Christiano that Herlitz junctional EB (JEB), for example, could be a difficult choice for therapy because of the potential multiplicity of defective components in the basal lamina that need correction. Using keratinocytes from a JEB patient, groups in Nice and Rome have demonstrated re-expression of laminin 5 with repair of hemidesmosomes and adhesion at the culture level, but this is still a long way from treatment regimes that might require correction of even more than one gene.

The immediate future exists in the development of vectors for delivery into autologous keratinocytes of a range of possible gene element(s) – be it a ribozyme to remove defective gene function, or replacing the defective gene with a normal one.

Effective therapy requires permanency through stem cells and stable gene insertions. Perhaps the real long-term future for all gene therapy, genodermatoses included, could reside in the relatively recent revelation that in enucleated oocytes, adult somatic chromatin can be dedifferentiated to pluripotentiality and reprogrammed to produce clones of an animal (1, 2).

What could be around the corner is a revolutionary method for the correction of mutations in keratinocytes *per se*, or the insertion of a desirable gene for other genetic reasons. It would be an *ex vivo*, cell therapy procedure and would involve biopsy and keratinocyte expansion in culture, dedifferentiation, homologous recombination with the normal gene, followed by reprogramming to the keratinocyte lineage and grafting to the patient.

Of course, we don't yet have the defined conditions for dedifferentiation *in vitro* that simulate what can be achieved in oocytes, and we don't know what degree of dedifferentiation would be adequate to carry out the scenario proposed. However, on present indications of known factors (e.g. LIF), the answers will surely eventuate, possibly quite rapidly. Biological factors of the opposite kind, that act in directing the differentiation of embryonic stem cells to primitive ectoderm, are already being isolated (3).

Finally, "the prevention better than therapy" argument is a sensible one, and the single-cell pre-implantation diagnostic technique on very early embryos is a striking advance. However, surely the difficulties here are the identification of possible carriers for testing by this procedure and the acceptability, on medical and ethical grounds, of obligatory *in vitro*-fertilization to those patients potentially at risk.

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Commentary 4

Epidermal gene therapy is indeed worth considering. However, especially when it comes to treatment of localized skin diseases, substantial hurdles were described, such as dominant mutations and a need for regulated expression. But then, will it be less problematic to use epidermal gene therapy to treat systemic disorders, such as metabolic diseases?

Sustained epidermal gene expression requires gene transfer into stem cells. Several studies have suggested that this might be possible (1–5), and the advances in characterization of these cells and in defining their location in the skin (6) will probably further enhance the possibilities of specific stem cell targeting.

Epidermal gene therapy with the purpose of achieving sustained gene expression has mostly been based on the use of retroviral vectors. Delivery of retroviral vectors directly to the skin (*in vivo* gene therapy) has been difficult to achieve, and therefore retrovirally mediated gene transfer into the skin has been performed *ex vivo* followed by grafting. Because of the technical difficulties and expenses associated with these procedures, methods allowing *in vivo* delivery followed by long term expression will be of great value. Chimeric viral vector systems that incorporate the favorable attributes of two different viral vectors, such as the efficient *in vivo* transduction properties of adenoviral vectors and the stable integration of retroviral vectors, might be a solution to these problems. Combination of viral and non-viral gene transfer methods, such as the use of plasmovirus (7), should also be considered.

Why consider epidermal gene therapy of metabolic diseases?

The skin is the largest organ in the body. Epidermal keratinocytes have a high metabolic capacity, they can readily be cultivated *in vitro* and trans-

Table 1.

Neo-organ	Metabolic sink
apoE	Ornithine aminotransferase
apoA1	Adenosine aminotransferase
Factor VIII	Phenylalanine hydroxylase
Factor IX	Ornithine transcarbamylase
Human growth hormone	LDL-receptor
Transferrin	
Insulin	
Erythropoietin	

planted back onto patients. The blood supply to the skin is considerable (8.5% of the cardiac output), it can be regulated, and it can exceed the demand at least 10 fold. For safety reasons, the skin is also very attractive, since genetically modified cells can be easily removed.

Two scenarios can be visualized, either the production of medically relevant proteins in keratinocytes that secrete them into the circulation epidermis as a "*neo-organ*" (8), or production of enzymes that can detoxify the body for toxic substances accumulating in certain disorders the "*metabolic sink approach*" (9) (Table 1).

For each application it is important to investigate in detail the molecular mechanisms. Consider the delivery of a protein to the circulation. What are the transport barriers? The efficiency of synthesis and secretion varies substantially among different proteins. The transport across the basement membrane probably depends on the size and the hydrophobicity of the protein. Also, the stability of the protein in the circulation is important to consider.

For the "metabolic sink" approach the situation is even more complicated.

As illustrated by the attempts to clear ornithine from the skin (10), the metabolic capacity of an epidermal graft depends not only on the amount of enzyme produced in the cells, but also on parameters such as co-factor supply, regional substrate concentrations and clearance of downstream metabolites. In fact, genetic manipulation at several points along the metabolic pathway will probably be necessary to achieve clinical success.

In spite of the challenges associated with gene therapy of skin diseases, there are no indications so far that systemic metabolic diseases will be a more straightforward target for epidermal gene therapy than classical genodermatoses.

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Commentary 5

The start of Molecular Pathology was in 1949 with the finding of a difference in the electrophoretic mobility of normal hemoglobin and sickle cell hemoglobin (HbS). In 1957, a valine for glutamic acid substitution in the beta-chain was determined to cause HbS with its abnormal physical properties. Sickle cell anemia is a serious disease and occurs in 0.15% of American blacks; 8% of American blacks are carriers. Yet, 50 years after determining the basis of the disease there is still no molecular therapy.

Bone marrow transplantation works. A ribozyme-based method for treatment has passed the proof of concept phase, but, still no magic bullet for a common and important disease. An interesting approach to therapy is using azacytidine or hydroxyurea to increase hemoglobin F, decreasing sickling and helping the disease. Sickle cell anemia is a powerful example that knowing the molecular cause does not rapidly lead to a molecular cure. Learning patience is hard.

Blackboard schemes of therapy should not lead to hyperbole and resulting hubris – this is the Faustian trap which has caught many well-intentioned scientists. One should not be surprised that many viral based vectors will lead to antibodies which may interfere with the treatment. Raising false hope can be even more destructive to the patient and their families than the ignorance of a disease.

These are some of the issues that require more consideration (1, 2).

- *Dominant Negative Mutations:* a serious issue – a little of a "bad molecule" can interfere with large amounts of a normal molecule, especially a problem with some cytokeratin mutations.

- *Stem Cell Targets:* if a gene is integrated into the more differentiated cells, repeated therapy is needed. This is not the end of the world – most drugs for most conditions require continued, albeit, intermittent administration. Stem cells may not be the real or the desired target – their turnover may be too low. Introduction into a cell like a transient amplifying cell may be better.
- *Change the mutations:* with point mutations there are drugs which can change the transcription of a nonsense or termination codon into a functional codon with therapeutic effect.
- *Regulate the molecule:* this approach is not valued as much as it should be. With the whole human genome available for transcription, there is the basis for increasing fetal, embryonic or geriatric transcripts which can change the physiology of the cell and its internal and secreted products. Growth factors such as TGF-beta and retinoids can change the ratio and transcription of specific gene products.

The important lesson is to not limit your thinking by considering "gene therapies" to mean only introducing genes. Think of changing the transcription of messengers, think inducing genes, which are not normally expressed at sufficient quantities to be therapeutic. Such changes may be using old style, low molecular weight drugs, antisense and triplex nucleotides, ribozymes and the like.

Hope – molecular biology gives hope for preventing serious diseases by diagnosis in the preimplantation stage as discussed by Dr Christiano, a powerful methodology.

If we fall prey to being mesmerized by molecular wizardry and scientific paraphernalia and lose the ability to think about attacking genetic diseases in all the ways possible, we have not benefited fully from the new technologies. The real new therapies will be elegant and use all of the molecular knowledge we have obtained about the skin and its genes. Let's continue working – patients are counting on us to fulfill our promises.

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Commentary 6

Remember the last of those nasty computer viruses, Melissa: one click on the attachment and all those post-doc-man-years of hard work disappeared, as it gobbled its way through your hard drive, lost for eternity. Well, there is a far more pernicious virion that has been spreading through the biomedical community for at least 10 years.

Seemingly invisible to the collective immune system, this destructive little creature, unblemished by logical assault, or rational drug design, has now spread to the lay public, grant givers, fund-raisers, and patient groups. This is the virus of gene therapy, and to understand its destructive force, to fathom its epidemiology, we need to understand its symbiosis with that icon of 20th-century biology, genetics.

Genetics has had two golden periods in the present century. The first being the re-discovery of Mendel's work, and the subsequent resolution of the treatment of what we would now call Mendelian disorders and complex (or let us say Gaussian traits) in the synthesis, by Fisher and others, that is now quantitative genetics. Then there is an interlude of say 50 years, broken only by Crick and Watson's discovery, before the technical outpourings of the last 2 decades, allowing the tracking of disease-associated genes. The technical facility of reverse genetics, together with the insights into the use of polymorphisms between subjects to track disease, by David Botstein and others, have provided disease researchers with a golden period of essentially molecular natural history: "molecular case reports" as Bert Vogelstein remarked of a major genetics journal.

The achievements of this approach, are either over-hyped beyond recognition – understandably, perhaps – or even more curiously underplayed. And the reasons for this latter aspect belie the problems of gene therapy. Thus, the importance of gene identification of Mendelian disorders, prognosis, classification, clinical bootstrapping in terms of syndrome identification and diagnostic acumen, or prenatal diagnosis and preventative intervention, are perhaps overlooked in the mad dash for therapy, and the delusion of the generic approach to medical science. Let me explain.

The strength of positional cloning and of much human genetics has been that, essentially, it has been a black box approach. Anonymous markers cosegregating with a phenotype can be tracked. Genes for a disease can be found in the complete absence of knowledge of pathophysiological mechanisms: a revolutionary concept that should still make us sit up and stare. Yet, progress from here is less generic, and here lies the hubris.

Genetics has wedded itself to big science, a form of physics envy, and wishes to drive its ambitious project forward in two directions, gene therapy, and the genetic elucidation of complex disease. Both projects remain at present triumphs of marketing over substance. It is as though everybody has started to believe those opening sentences of their own grant applications.

Yet history suggests that the golden period of physics ended with the transition to big science, and that the most interesting areas of the hard sciences have been relocated to former intellectual backwaters. Such will be the fate of this new biology. Whereas positional cloning has enjoyed the generic nature of its activity – just show a pedigree to a human geneticist, who cares less about context or how you spell the name of the disease – there is no logical process to proceed to therapy from gene identification. Medicine remains a branch of applied biology, opportunists standing on the backs of medicinal chemistry.

This isn't to deny a role for gene-based therapy. I think there will be such niches, and these avenues of opportunity should be exploited. It is just that, at present, the subject shares the intellectual finesse of somebody, who, aware of Pasteur's elucidation of the infectious nature of disease, seeks to preempt the next century of discovery by having "discovered, that most microbes are heat sensitive" announces – by press release closely followed by stock market flotation – that man's fight with infectious disease is over. Delivery of destruction, friendly fire, acceptability, limited collateral damage, to use military terms, or even biological plausibility are all given short shrift!

So how do we go forward? First a little realism. Impact factors, and glossy covers don't match the recent (albeit incremental) successes of phototherapy, topical immunosuppression or the rediscovery of the infectious nature of some forms of eczema. Second, in English at least, the political use of the word revolution comes from Galileo's use of the same word to describe the motion of the planets. Science needs to remain a revolutionary activity: forget grand strategies, 5-year plans, post genome-integrative projects to ensure full employment of geneticists. *Think small.* As the poet and mystic William Blake understood, if you want to do good, do it in the study of those "minute particulars".

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